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FORM PTO-1390 (REV 1-98) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 3339-392

U S APPLICATION NO (If known, see 37 C F R. 1 5): 09/254,032

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR97/01541	SEPTEMBER 1, 1997	AUGUST 30, 1996

TITLE OF INVENTION

ANTIGENS DERIVED FROM FILAGGRIN AND THEIR USE FOR DIAGNOSING RHEUMATOID POLYARTHRITIS

APPLICANT(S) FOR DO/EO/US

GUY SERRE, ET AL.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☐ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☒ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l).

4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☐ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ has been transmitted by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ have been transmitted by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. To 16. Below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.

12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information: English version of form PCT/IPEA/409

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U.S. APPLICATION NO (If known, see 37 C F R 1 50)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/254,032	PCT/FR97/01541	3339-392

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$970.00**		
International preliminary examination fee (37 CRF 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$840.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search (37CFR 1.445(a)(2)) paid to USPTO **$760.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO But all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$ 96.00**		
ENTER APPR0PRIATE BASIC FEE AMOUNT =	**$**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	**$ 130.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	0 -20 =	0	X $18.00	$ 0.00	
Independent Claims	0 - 3 =	0	X $78.00	$ 0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $260.00	$ 0.00	
TOTAL OF ABOVE CALCULATIONS =				$ 130.00	
Reduction by ½ for filing by small entity, if applicable. A Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$ 130.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$ 0.00	
TOTAL NATIONAL FEE =				$ 130.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property+				$ 40.00	
TOTAL FEES ENCLOSED =				$ 170.00	
				Amount to be Refunded	$
				Charged	$

a. ☒ A check in the amount of $ 170.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 16-0605 in the amount of $ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-0605. A duplicate copy of this sheet is enclosed.

Note: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

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Date of Deposit: April 26, 1999

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to BOX PCT, Attn: DO/US (PTO) Assistant Commissioner for Patents, Washington, DC 20231.

DENISE G. REAVES

IN THE UNITED STATES DESIGNATED OFFICE (DO/US)

In re:	Guy Serre, et al.	Attn: **DO/US**
International Appl. No.:	PCT/FR97/01541	February 26, 1999
International Filing Date:	September 1, 1997	
For:	ANTIGENS DERIVED FROM FILAGGRIN AND THEIR USE FOR DIAGNOSING RHEUMATOID POLYARTHRITIS	

Box PCT
Assistant Commissioner of Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the above-identified application as follows:

In The Claims:

Please note that the International Preliminary Examination Report indicates that the claims have been amended. It is the understanding of applicant's representative that claims 1-8 on the two numbered pages 22 and 23 labeled as "Amended Sheet" are the English language claims corresponding to the French language claims which were amended during the International Preliminary Examination, and are the claims that are now before the Examiner. It is requested that the Preliminary Amendments set forth below be applied to those claims.

In claim 7, line 5 and 6, delete “or an antigenic composition as claimed in claim 6,".

In claim 7, line 10 delete "by any appropriate means,".

In claim 8, line 4 and 5, delete “or an antigenic composition as claimed in claim 6,"

In claim 8, line 8 and 9, please delete ", and/or means for detecting said antigen/antibody complex".

Please add the following new claims:

9. A method of detecting the autoantibodies specific for rheumatoid arthritis in a biological sample, which method comprises:

- bringing said biological sample into contact with an antigenic composition as claimed in claim 6, under conditions allowing the formation of an antigen/antibody complex with the autoantibodies specific for rheumatoid arthritis which may be present;
- detecting the antigen/antibody complex which may be formed.

10. A kit for the detection of autoantibodies specific for rheumatoid arthritis in a biological sample, which comprises at least one antigenic composition as claimed in claim 6, as well as buffers and reagents appropriate for constituting a reaction medium allowing the formation of an antigen/antibody complex.

REMARKS

The above amendments are made to more clearly define the invention under United States practice. Please enter this amendment prior to calculation of the filing fee.

Respectfully submitted,

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DENISE G. REAVES

ANTIGENS DERIVED FROM FILAGGRINS AND THEIR USE FOR THE DIAGNOSIS OF RHEUMATOID ARTHRITIS

The present invention relates to new preparations of antigens specifically recognized by autoantibodies specific for rheumatoid arthritis.

Rheumatoid arthritis (hereinafter abbreviated "RA") is the most frequent of the chronic inflammatory rheumatisms. It is an autoimmune disease, and the serum of affected patients contains autoantibodies of which some are specific, and may constitute a marker for this disease, allowing its diagnosis even at early stages. Research studies have therefore been carried out in order to identify antigens recognized by these antibodies, in order to obtain therefrom purified preparations which can be used in conventional immunological diagnostic techniques.

Autoantibodies which are specifically present in patients suffering from RA and which react with a rat esophageal epithelial antigen were described for the first time by B.J.J. Young et al. in Br. Med. J. 2:97-99, (1979). These autoantibodies were at the time called "antikeratin antibodies".

During previous studies, the inventors' team obtained, from human and murine malpighian epithelia, preparations of antigens related to filaggrin and to profilaggrin, which are specifically recognized by the antibodies present in the serum of patients suffering from rheumatoid arthritis, and showed that the "antikeratin antibodies" were in fact anti-filaggrin autoantibodies (hereinafter called "AFA"). Application EP 0,511,116 describes these antigenic preparations and their use for the diagnosis of rheumatoid arthritis.

Filaggrins are a family of proteins which has been identified in various species, inter alia in humans, rats, mice, guinea pigs, at the level of the keratinizing malpighian epithelia [for a review on filaggrins, cf. DALE et al. [The Keratinocyte Handbook, Cambridge University Press, pp 323-350, (1994)]. They

are derived from the dephosphorylation and from the proteolysis of a precursor, profilaggrin, which essentially consists of repeated domains of filaggrin separated by interdomain peptide segments.

The gene encoding profilaggrin is composed of repeating subunits each of which encodes a molecule of filaggrin, which are separated by portions encoding the interdomain peptide segments. All the repeating units encoding each of the human filaggrins have the same length (972 base pairs in humans); however, in humans, large (10-15%) sequence variations are observed from one subunit to another. While the majority are conservative, some of these variations induce changes in amino acids and in some cases changes in the electrical charge on the protein. Thus, human filaggrins form, independently of the post-transcriptional modifications, a heterogeneous population of molecules with a similar size but with different sequences and charges (pHi equal to 8.3 ± 1.1) [GAN et al., Biochem. 29, p. 9432-9440 (1990)].

Profilaggrin is a protein with a high molecular weight (about 400,000 in humans) which is soluble in the presence of high salt or urea concentrations. It has a high content of basic amino acids (arginine and histidine), as well as of glycine, serine and glutamic acid. It is low in nonpolar amino acids and does not contain methionine, cysteine or tryptophan. It is highly phosphorylated on serine residues, which confers on it an isoelectric point close to neutrality.

Profilaggrin is cleaved into filaggrin units during a complex process of maturation involving dephosphorylation, followed by cleavage by proteases at the level of the interdomain segments. This cleavage first generates fragments of intermediate size, and then the functional molecules of filaggrin.

The filaggrins derived from the dephosphorylation and cleavage of profilaggrin are basic proteins whose content of amino acids is similar

to that of the profilaggrins. They participate in the organization of the keratin filaments and undergo gradual maturation during which the basic arginine residues are converted to neutral citrulline residues under the action of peptidylarginine deiminase [HARDING C.R. and SCOTT I.R., J. Mol. Biol. 170, p. 651-673 (1983)]. This causes a reduction in their affinity for the keratins from which they become detached; they are then completely degraded under the action of various proteases.

The properties of filaggrins and profilaggrins have been particularly well studied in rats, in mice and in humans. The size of profilaggrin varies, depending on the species, from 300 to 400 kD and that of the filaggrins from 27 to 64 kD.

The polymorphism observed in humans between the sequences of filaggrin units within the same profilaggrin gene does not appear in rats and mice. The filaggrins exhibit, in addition, a high inter- and intra-specific variability at the level of their sequence. This variability does not however affect their functional properties or their overall amino acid composition, and their biochemical properties. Likewise, the tissue locations of profilaggrin and of filaggrins are identical in the various mammals studied.

Continuing their studies, the inventors observed that the profilaggrin present in the keratohyalin granules of the human epidermis was not, contrary to the filaggrins, recognized by AFAs [SIMON et al. Clin. Exp. Immunol. 100, 90-98 (1995)]. They then tested the reactivity of the AFAs with recombinant filaggrin, and observed that the latter was not recognized either. On the other hand, it had been previously observed that the forms of the human epidermal filaggrins mainly recognized by the AFAs were the acido-neutral forms described by SIMON et al. [J. Clin. Invest., 92, 1387, (1993)] and in application EP 0,511,116. The fact that these acido-neutral forms

correspond to a late stage of maturation of filaggrin made it possible to suppose that all or part of the post-translational modifications occurring up to this stage were involved in the formation of the epitopes recognized by the AFAs.

To verify this hypothesis, the inventors sought to reproduce *in vitro*, using recombinant filaggrin, these post-translational modifications in order to determine the ones which were capable of influencing the antigenicity of filaggrin.

They thus observed that in fact the citrullination of filaggrin was sufficient to generate epitopes recognized by the AFAs. Indeed, they observed, by carrying out the deimination *in vitro* of recombinant filaggrin, that the replacement of at least part of the arginines with citrullines allows an antigen to be obtained which is specifically recognized by the AFAs present in the serum of patients suffering from RA.

The subject of the present invention is an artificial antigen which is specifically recognized by the AFAs present in the serum of patients suffering from RA, characterized in that it consists of a recombinant or synthetic polypeptide comprising all or part of a sequence derived from that of a filaggrin unit or of a related molecule, by replacing at least one arginine residue with a citrulline residue. Preferably, an antigen in accordance with the invention comprises at least 5 consecutive amino acids, and advantageously at least 10 consecutive amino acids, including at least one citrulline, of said sequence.

For the purposes of the present invention, "filaggrin unit" is understood to mean a polypeptide whose sequence is that of the product of translation of any one of the subunits encoding a filaggrin domain of the gene for human profilaggrin or from another species, or alternatively is a consensus sequence, a theoretical sequence obtained from the sequences of the filaggrin domains.

For the purposes of the present invention, "related molecule" is understood to mean any molecule having at least one arginine residue capable of being converted to a citrulline residue under the action of a PAD (peptidylarginine deiminase); by way of example, this PAD may be a rabbit muscle PAD, as shown in the examples below. It is however within the capability of persons skilled in the art to select any other appropriate PAD by simple routine tests, by reacting it with noncitrullinated human filaggrin.

The term "peptide" as used in the present application means in particular protein or protein fragment, oligopeptide, extracted, separated or substantially isolated or synthesized, especially those obtained by chemical synthesis or by expression in a recombinant organism; any peptide in whose sequence one or more amino acids of the L series are replaced by an amino acid of the D series, or vice versa; any peptide in which at least one of the CO-NH bonds, and advantageously all the CO-NH bonds of the peptide chain is (are) replaced with one or more NH-CO bonds; any peptide in which at least one of the CO-NH bonds and advantageously all the CO-NH bonds is or are replaced by one or more NH-CO bonds, the chirality of each aminoacyl residue, whether it is involved or not in one or more abovementioned CO-NH bonds, being either conserved or reversed in relation to the aminoacyl residues constituting a reference peptide, these compounds being also designated immunoretroids, a mimotope, and the like.

Antigens in accordance with the invention may for example be obtained by the action of PAD on natural, recombinant or synthetic peptides or proteins comprising arginine residues; they may also be obtained by peptide synthesis by directly incorporating one or more citrulline residues into the synthesized peptide.

According to a preferred embodiment of an antigen in accordance with the present invention, it consists of a polypeptide comprising all or part of the

sequence corresponding to amino acids 144 to 314 of a human filaggrin unit, in which at least one arginine residue has been replaced by a citrulline residue, or alternatively all or part of the sequence corresponding to amino acids 76 to 144 of a human filaggrin unit, in which at least one arginine residue has been replaced with a citrulline residue.

An antigen in accordance with the invention may for example consist of a peptide comprising all or part of the sequence corresponding to amino acids 71 to 119 or a human filaggrin unit, in which at least one arginine residue has been replaced with a citrulline residue.

Advantageously, an antigen in accordance with the invention consists of a peptide comprising all or part of at least one sequence derived from one of the sequences identified in the sequence listing in the annex under the numbers SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 6, by replacing at least one arginine residue with a citrulline residue.

The subject of the present invention is also the use of the antigens in accordance with the invention, as defined above, for the *in vitro* diagnosis of RA.

The present invention covers in particular antigenic compositions for diagnosing the presence of autoantibodies specific for RA in a biological sample, which compositions are characterized in that they contain at least one antigen in accordance with the invention, optionally labeled and/or conjugated with a carrier molecule.

The subject of the present invention is also a method of detecting class G autoantibodies specific for RA in a biological sample, which method is characterized in that it comprises:

- bringing said biological sample into contact with at least one antigen in accordance with the invention, as defined above, under conditions allowing the formation

of an antigen/antibody complex with the autoantibodies specific for RA which may be present;

- detecting, by any appropriate means, the antigen/antibody complex which may be formed.

This method of detection may be carried out using a kit comprising at least one antigen according to the invention, as well as buffers and reagents appropriate for constituting a reaction medium allowing the formation of an antigen/antibody complex, and/or means for detecting said antigen/antibody complex.

Said kit may also comprise, where appropriate, reference samples, such as one or more negative sera and one or more positive sera.

The present invention will be understood more clearly with the aid of the additional description which follows, which refers to examples of preparation and use of antigens in accordance with the invention.

EXAMPLE 1 : REACTIVITY OF SERA OBTAINED FROM PATIENTS SUFFERING FROM RHEUMATOID ARTHRITIS ON EPIDERMAL FILAGGRINS

A piece of human epidermis is ground with the aid of a "Potter" type electric grinder in a buffer with a high urea concentration (6 M), which makes it possible to solubilize all the epidermal filaggrins.

With this epidermal extract, two-dimensional electrophoresis (8-25% acrylamide gel in the presence of 6 M urea) is carried out; the 1st dimension corresponds to a gel isoelectrofocusing in a pH gradient ranging from 5 to 8 and the second dimension corresponds to electrophoresis under denaturing conditions, in the presence of SDS. After electrophoresis, the proteins in the gel are transferred onto nitrocellulose.

The immunological reactions are carried out according to a conventional protocol.

The nitrocellulose membrane is incubated overnight at 4°C with a serum from a patient suffering from RA, diluted 1/2000, and then the serum immunoglobulins which have reacted with the antigens

bound to the membrane are detected with the aid of a peroxidase-labeled anti-human IgG secondary antibody. The presence of the peroxidase substrate is revealed by the ECL (Enhanced ChemiLuminescence, AMERSHAM) method according to the protocol recommended by the manufacturer.

In a second stage, the same membrane is washed and then incubated for one and a half hours at 20°C in the presence, this time, of the monoclonal antibody AHF1 described by SIMON et al. [J. Invest. Dermatol. 105, 432, (1995)] at a concentration of 0.2 µg/ml, and then of a peroxidase-labeled anti-mouse IgG secondary antibody. The reaction is revealed by the ECL method, as indicated above.

The results are illustrated by Figure 1:

The monoclonal antibody AHF1 recognizes isoforms of filaggrin whose pHi ranges from 5.8 to 8.5. On the other hand, only the isoforms whose pHi ranges from 5.8 to 7.4 are detected by the serum from the patient suffering from RA.

The fact that only the most acidic isoforms of filaggrins are detected makes it possible to assume that the acidification of these isoforms forms part of the post-translational modifications which would be necessary for the recognition of filaggrin by the antibodies present in the sera from patients suffering from RA.

EXAMPLE 2 : *IN VITRO* DEIMINATION OF RECOMBINANT FILAGGRIN BY PEPTIDYLARGININE DEIMINASE (PAD)

Recombinant filaggrin is produced according to the following protocol:

A DNA fragment encoding a filaggrin unit is amplified by PCR, using human genomic DNA (RAJI cells: ATCC CCL86) with the aid of the following 2 primers:

5′ primer:

5′ TTCCTATACCAGGTGAGCACTCAT 3′

3′ primer:

5′ AGACCCTGAACGTCCAGACCGTCCC 3′

The amplification product is cloned into the SmaI site of the vector pUC19. The recombinant clones are selected by verifying the presence of a 972 bp insert obtained after digestion with SacI and XbaI. This insert is then subcloned into pUC19. The insert resulting from this subcloning is then transferred into the vector pGEX (marketed by the company PHARMACIA), between the EcoRI and HindIII sites. The expression vector thus obtained expresses, in *E. coli*, filaggrin fused with glutathione S-transferase (GST), under the control of the prokaryotic Tac promoter. The synthesis of the recombinant protein is induced by addition of isopropyl-β-D-galactoside (IPTG) to the culture.

The recombinant filaggrin thus obtained will be called hereinafter: "fil-gst".

The existence of 9 fragments which result from post-translational proteolysis of the full-length filaggrin is observed after electrophoresis. The positions of the various cuts generating these fragments are indicated in Figure 2.

The mixture of the 9 fragments is subjected to deimination *in vitro* by peptidylarginine deiminase.

A rabbit muscle peptidylarginine deiminase preparation (681 U/ml) marketed by TAKARA BIOMED EUROPE is used according to the protocol recommended by the manufacturer.

The operating conditions are the following:

- reaction medium: 0.1 M Tris-HCl, 10 mM $CaCl_2$, 5 mM DTT, pH 7.4;
- enzyme/substrate ratio: 140 mU/μmol of filaggrin containing 10% arginine or 4 mU/μmol of arginine;
- incubation: between 0 and 60 min at 50°C;
- termination of the reaction: heating 3 min in LAEMMLI buffer.

The following 8 reactions are carried out in parallel.

(1) BSA (bovine serum albumin) incubated in reaction medium (1 h, 50°C) in PAD.

(2) BSA incubated in reaction medium (1 h, 50°C) with 60 mU of PAD.

(3) fil-gst incubated in reaction medium (1 h, 50°C) without PAD.

(4) fil-gst incubated in reaction medium (5 minutes at 50°C) with 60 mU of PAD.

(5) fil-gst incubated in reaction medium (15 minutes at 50°C) with 60 mU of PAD.

(6) fil-gst incubated in reaction medium (30 minutes at 50°C) with 60 mU of PAD.

(7) fil-gst incubated in reaction medium (1 h at 50°C) with 60 mU of PAD.

(8) fil-gst incubated in reaction medium (1 h at 50°C) with 60 mU of PAD and in the presence of 10 mM N-ethylmaleimide (PAD inhibitor).

1 µl of each sample is deposited on an electrophoresis gel (PHAST®-SDS gel 12.5%, PHARMACIA), and the electrophoresis is carried out with the PHAST-SYSTEM® apparatus (PHARMACIA), under the conditions recommended by the manufacturer. After transfer onto nitrocellulose, the revealing is carried out either with a pool of 5 sera from patients suffering from RA, diluted 1/2000 (Figure 3a), or with the anti-filaggrin monoclonal antibody AHF2 [SIMON et al. J. Invest. Dermatol. 105, 432, (1995)] at the concentration of 0.2 µg/ml (Figure 3b).

The antigen/antibody complex is revealed with the aid of a peroxidase-coupled secondary antibody by the ECL technique.

The results are illustrated by Figure 3

Lane 1 : BSA (1 hour, 50°C)
Lane 2 : BSA + PAD (1 hour, 50°C)
Lane 3 : fil-gst (1 hour, 50°C)
Lane 4 : fil-gst + PAD (5 minutes, 50°C)
Lane 5 : fil-gst + PAD (15 minutes, 50°C)
Lane 6 : fil-gst + PAD (30 minutes, 50°C)
Lane 7 : fil-gst + PAD (1 hour, 50°C)
Lane 8 : fil-gst + PAD + inhibitor (1 hour, 50°C)

In the absence of a citrullination reaction, the fil-gst is not recognized by the sera from patients suffering from RA (Figure 3a, lane 3), whereas from 5 minutes of citrullination (Figure 3a, lane 4), it is detected by these sera. An increase in reactivity is observed with the pool of sera when PAD is reacted for 60 minutes at 50°C (Figure 3a, lane 7).

- fragments 1, 2, 3 (bands identified by points) of the fil-gst are strongly recognized, after citrullination, by the sera from patients suffering from RA. Fragments 4 and 5 (bands identified by asterisks) are also recognized. These results make it possible to assume that one or more epitopes with a high affinity exist in the COOH-terminal half of filaggrin (144 to 314), this epitope being repeated between positions 76 and 144.

- the monoclonal antibody AHF2 recognizes all the fragments of fil-gst, citrullinated or otherwise.

EXAMPLE 3 : SPECIFICITY OF THE RECOGNITION OF CITRULLINATED FIL-GST BY THE SERA

In a first series of experiments (Figure 4a), the reactivity of noncitrullinated fil-gst (fil-gst alone, 30 minutes at 50°C) and fil-gst citrullinated with PAD 30 minutes at 50°C is compared with human sera composed of:

- sera from normal persons: T(2) and T(3)

- sera from patients suffering from RA having high AFA titers which are detected by immunotransfer on acido-neutral variants of human filaggrin, and by indirect immunofluorescence on cryosections of rat esophagus: RA(6) and RA(8);

- anti-filaggrin antibodies purified from the serum from a patient suffering from RA by affinity chromatography, on a column grafted with acido-neutral isoforms of human filaggrin: AFA.

A positive control is also carried out with the monoclonal antibody AHF2.

In a second series of experiments (Figure 4b), the reactivity of citrullinated fil-gst is confirmed with a larger series of sera:

- 4 control sera: T(4)
- 4 sera from patients suffering from RA not having AFAs detectable by immunotransfer or by indirect immunofluorescence: RA(4)
- 9 sera from patients suffering from RA with high AFA titers (three of them (*) were also tested in the first series of experiments): RA(9)
- anti-filaggrin antibodies purified by affinity chromatography, on a column grafted with the acido-neutral isoforms of filaggrin, from a pool of sera from 40 patients suffering from RA: AFA
- the monoclonal antibodies AHF (1-7).

The sera were used at the dilution of 1/2000; the anti-filaggrin antibodies purified by affinity chromatography are used at the concentration of 4 µg/ml; the monoclonal antibodies are used at the concentration of 0.2 µg/ml.

The results are the following:

- the citrullination of recombinant filaggrin is necessary for the recognition by the AFAs of the sera from patients suffering from RA (14 positive sera out of 14 recognize it);
- the antifilaggrin autoantibodies, purified by affinity chromatography from the sera from patients suffering from RA, show the same reactivity on citrullinated fil-gst as the sera from patients suffering from RA (recognition of the fragments corresponding to lanes 1 to 5). This shows that it is indeed the AFAs present in these sera which recognize the citrullinated fil-gst.

EXAMPLE 4 : CITRULLINATION OF THE PEPTIDES S-47-S AND S-35-R BY PAD, AND TEST OF THE REACTIVITY OF THE CITRULLINATED PEPTIDES.

The peptide of 49 amino acids S-47-S having the sequence (1-letter code):

NH_2-STGHSGSQHSHTTTQGRSDASRGSSGSRSTSRETRDQEQSGDGSRHSGS-COOH

corresponding to amino acids 71 to 119 of the sequence of a human filaggrin unit, and comprising 6 arginine residues, and

the peptide of 37 amino acids S-35-R having the sequence (1-letter code):

NH_2-SQDRDSQAQSEDSERRSASASRNHRGSAQEQSRDGSR-COOH

corresponding to amino acids 155 to 191 of the sequence of a human filaggrin unit, and comprising 7 arginine residues, were prepared by peptide synthesis. The peptides S-47-S and S-35-R are represented in the sequence listing in the annex under the respective numbers SEQ ID NO: 3 and SEQ ID NO: 4.

These 2 peptides, as well as fil-gst, were citrullinated by the action of PAD, for 30 minutes at 50°C, in the same reaction medium as that indicated in Example 2. The specific conditions for each peptide, and for the fil-gst are the following:

- peptide S-47-S: 4 mU/μmol arginine
- peptide S-35-R: 2.7 mU/μmol arginine
- fil-gst: as indicated in Example 2.

The reactivity of each peptide and that of fil-gst, before and after action of the enzyme, towards the monoclonal antibody AHF4, and the serum from a patient suffering from RA, is compared by dot-blot.

The operating conditions are the following:

- 0.5 μg by deposition of each antigen (peptides, fil-gst, acido-neutral variants of filaggrin (AVF))
- nitrocellulose treatment 45 minutes at 80°C, before immunodetection
- RA serum used at the dilution of 1/2000; monoclonal antibody AHF4 used at a concentration of 0.2 μg/ml

The results are illustrated by Figure 5, which shows that:

- AHF4 recognizes the peptide S-47-S and fil-gst, citrullinated or not, but does not recognize S-35-R, citrullinated or not.

- S-47-S is recognized, after citrullination, by the serum from the patient suffering from RA, whereas S-35-R, citrullinated or not, is not recognized. The same serum recognizes, moreover, the AVFs and the citrullinated fil-gst but does not recognize the noncitrullinated fil-gst.

EXAMPLE 5 : SYNTHESIS OF THE PEPTIDES E-12-H AND E-12-D CITRULLINATED AND NONCITRULLINATED AND TEST OF THE REACTIVITY OF THE PEPTIDES.

The peptides E-12-H and E-12-D were determined with reference to the nucleotide sequences of the gene for human profilaggrin which are described by GAN S.Q et al. [Biochemistry, 29: 9432-9440, (1990)].

The peptide of 14 amino acids E-12-H having the sequence (1-letter code):

NH_2-EQSADSSRHSGSGH-COOH

comprises 1 arginine residue, and

the peptide of 14 amino acids E-12-D having the sequence (1-letter code):

NH_2-ESSRDGSRHPRSHD-COOH

comprises 3 arginine residues.

The peptides E-12-H and E-12-D are represented in the sequence listing in the annex under the respective numbers SEQ ID NO: 5 and SEQ ID NO: 6.

These peptides were prepared by solid phase peptide synthesis.

The citrullinated peptides E-12-H and E-12-D were directly synthesized by incorporation of a citrulline by replacing an arginine.

For the peptide E-12-D, only the arginine residue corresponding to the 8th amino acid of the sequence was replaced by a citrulline during peptide synthesis.

The reactivity of each citrullinated and noncitrullinated peptide was tested respectively in relation to a normal serum, to two sera from RA patients, to anti-filaggrin antibodies (AFAs) purified from a pool of 45 sera from RA patients and to anti-

filaggrin antibodies purified from 12 sera from RA patients.

EXPERIMENTAL PROTOCOL:

The wells of NUNC MAXISORP microtiter plates were respectively coated with the aid of the noncitrullinated and citrullinated peptides E-12-D and E-12-H, diluted to a concentration of 5 µg/ml in a PBS buffer (pH: 7.4) and incubated overnight at 4°C (final volume: 100 µg/well). The wells were saturated for 30 minutes at 37°C in PBS-Tween 20, 0.05%, 2.5% gelatin, 200 µl/well. The negative control serum (normal serum) was diluted 1/120. The anti-filaggrin antibodies were diluted in PBS-Tween 20, 0.05% - 0.5% gelatin (PBS TG) such that the final anti-filaggrin autoantibody concentrations are those indicated in the accompanying Table I. The negative control serum, the RA sera and the anti-filaggrin antibodies were added (final volume: 100 µl/well) and incubated for 1 hour at 37°C and overnight at 4°C. Peroxidase-labeled goat antibodies anti-gamma heavy chains of the human immunoglobulins (marketed by the company SOUTHERN BIOTECHNOLOGIES) were added to each well (dilution in PBSTG: 1/2000, final volume: 100 µl/well) and incubated for 1 hour at 37°C. The revealing was carried out by addition of ortho-phenylenediamine (2 mg/ml, for 10 minutes).

The results presented in the accompanying Table I are given as a ratio of OD at 492 nm: citrullinated peptide signal/noncitrullinated peptide signal.

These results show that in the majority of cases, the citrullinated peptide/noncitrullinated peptide OD ratio is greater than 1, and therefore illustrate the good sensitivity of the citrullinated peptides compared with the non-citrullinated peptides for their reactivity toward the anti-filaggrin autoantibodies.

TABLE 1

Peptide	Control serum	RA1 serum		RA2 serum			Pool of AFAs			AFAs purified from 12 RA sera											
		10*	20*	5*	10*	20*	5*	10*	20*	10*	10*	10*	10*	10*	10*	10*	10*	10*	10*	10*	10*
E-12-D	1.076	1.42	1.85	2.42	3.77	5.57	1.77	1.63	1.48	1.99	1.38	2.48	1.19	1.12	3.50	1.87	5.19	1.13	1.57	1.11	1.65
E-12-H	1	1.32	1.20	10.44	11.51	8.38	2.45	2.42	1.82	7.16	2.05	1.06	1.18	0.76	13.57	4.14	3.18	1.14	3.66	1.22	5.84

*: Concentration of AFAs in μg/ml.

SEQUENCE LISTING

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(A) NAME: ARNAUD Michel
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(A) NAME: JOLIVET Michel
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(C) CITY: BRON
(E) COUNTRY: FRANCE
(F) POSTAL CODE: 69500

(ii) TITLE OF THE INVENTION: ANTIGENS DERIVED FROM FILAGGRINS AND THEIR USE FOR THE DIAGNOSIS OF RHEUMATOID ARTHRITIS

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTCCTATACC AGGTGAGCAC TCAT 24

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AGACCCTGAA CGTCCAGACC GTCCC 25

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ser Thr Gly His Ser Gly Ser Gln His Ser His Thr Thr Thr Gln Gly
1 5 10 15
Arg Ser Asp Ala Ser Arg Gly Ser Ser Gly Ser Arg Ser Thr Ser Arg
20 25 30
Glu Thr Arg Asp Gln Glu Gln Ser Gly Asp Gly Ser Arg His Ser Gly
35 40 45
Ser

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ser Gln Asp Arg Asp Ser Gln Ala Gln Ser Glu Asp Ser Glu Arg Arg
1 5 10 15
Ser Ala Ser Ala Ser Arg Asn His Arg Gly Ser Ala Gln Glu Gln Ser
20 25 30
Arg Asp Gly Ser Arg
35

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Glu Gln Ser Ala Asp Ser Ser Arg His Ser Gly Ser Gly His
1 5 10

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Glu Ser Ser Arg Asp Gly Ser Arg His Pro Arg Ser His Asp
1 5 10

CLAIMS

1. An artificial antigen which is specifically recognized by the antifilaggrin autoantibodies present in the serum of patients suffering from rheumatoid arthritis, which consists of a recombinant or synthetic polypeptide comprising all or part of a sequence derived from that of a filaggrin unit, by replacing at least one arginine residue with a citrulline residue.

2. The artificial antigen as claimed in claim 1, which consists of a peptide comprising all or part of at least one sequence derived:

- from the sequence corresponding to amino acids 144 to 314 of a human filaggrin unit, or alternatively

- from the sequence corresponding to amino acids 76 to 144 of a human filaggrin unit,

by replacing at least one arginine residue with a citrulline residue.

3. The artificial antigen as claimed in claim 2, which consists of a peptide comprising all or part of at least one sequence derived from the sequence corresponding to amino acids 71 to 119 of a human filaggrin unit, by replacing at least one arginine residue with a citrulline residue.

4. The artificial antigen as claimed in claim 1, which consists of a peptide comprising all or part of at least one sequence derived from one of the sequences SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 6, by replacing at least one arginine residue with a citrulline residue.

5. Use of the antigen as claimed in any one of claims 1 to 4 for the *in vitro* diagnosis of rheumatoid arthritis.

6. An antigenic composition for diagnosing the presence of autoantibodies specific for rheumatoid arthritis in a biological sample, which contains at

least one antigen as claimed in any one of claims 1 to 4, optionally labeled and/or conjugated with a carrier molecule, with the exclusion of compositions with a structure identical to that of a preparation of isoforms of filaggrin which is purified from the human epidermis comprising a mixture of isoforms having a molecular weight of 40,000 and a pI ranging between 5.8 and 7.4.

7. A method of detecting the autoantibodies specific for rheumatoid arthritis in a biological sample, which method comprises:

- bringing said biological sample into contact with an antigen as claimed in any one of claims 1 to 4, or an antigenic composition as claimed in claim 6, under conditions allowing the formation of an antigen/antibody complex with the autoantibodies specific for rheumatoid arthritis which may be present;
- detecting, by any appropriate means, the antigen/antibody complex which may be formed.

8. A kit for the detection of autoantibodies specific for rheumatoid arthritis in a biological sample, which comprises at least one antigen as claimed in any one of claims 1 to 4, or an antigenic composition as claimed in claim 6, as well as buffers and reagents appropriate for constituting a reaction medium allowing the formation of an antigen/antibody complex, and/or means for detecting said antigen/antibody complex.

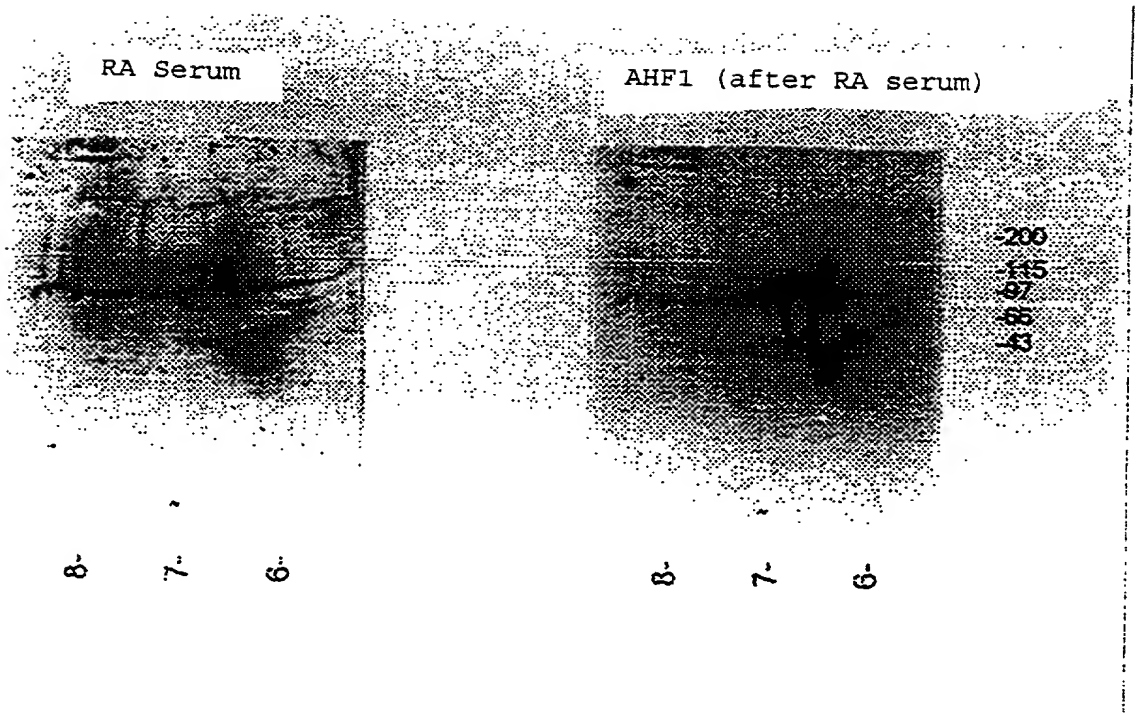

FIG.1

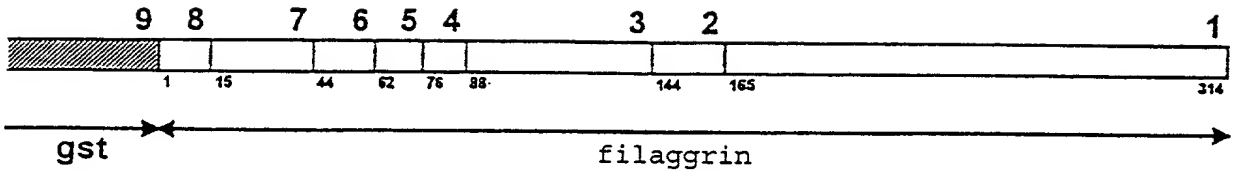

FIG.2

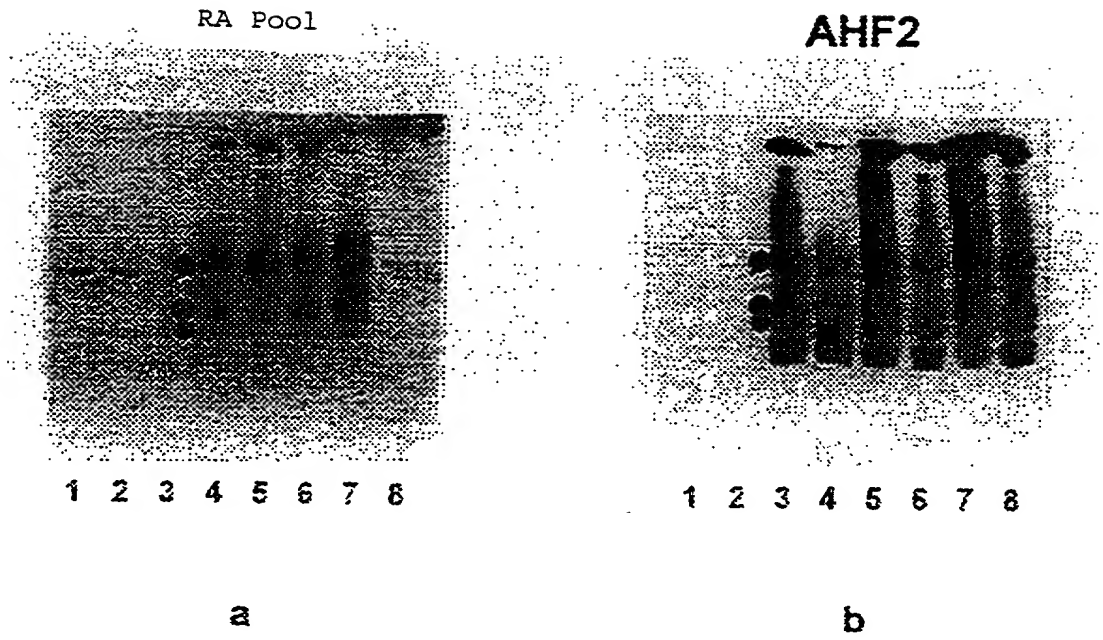

FIG.3

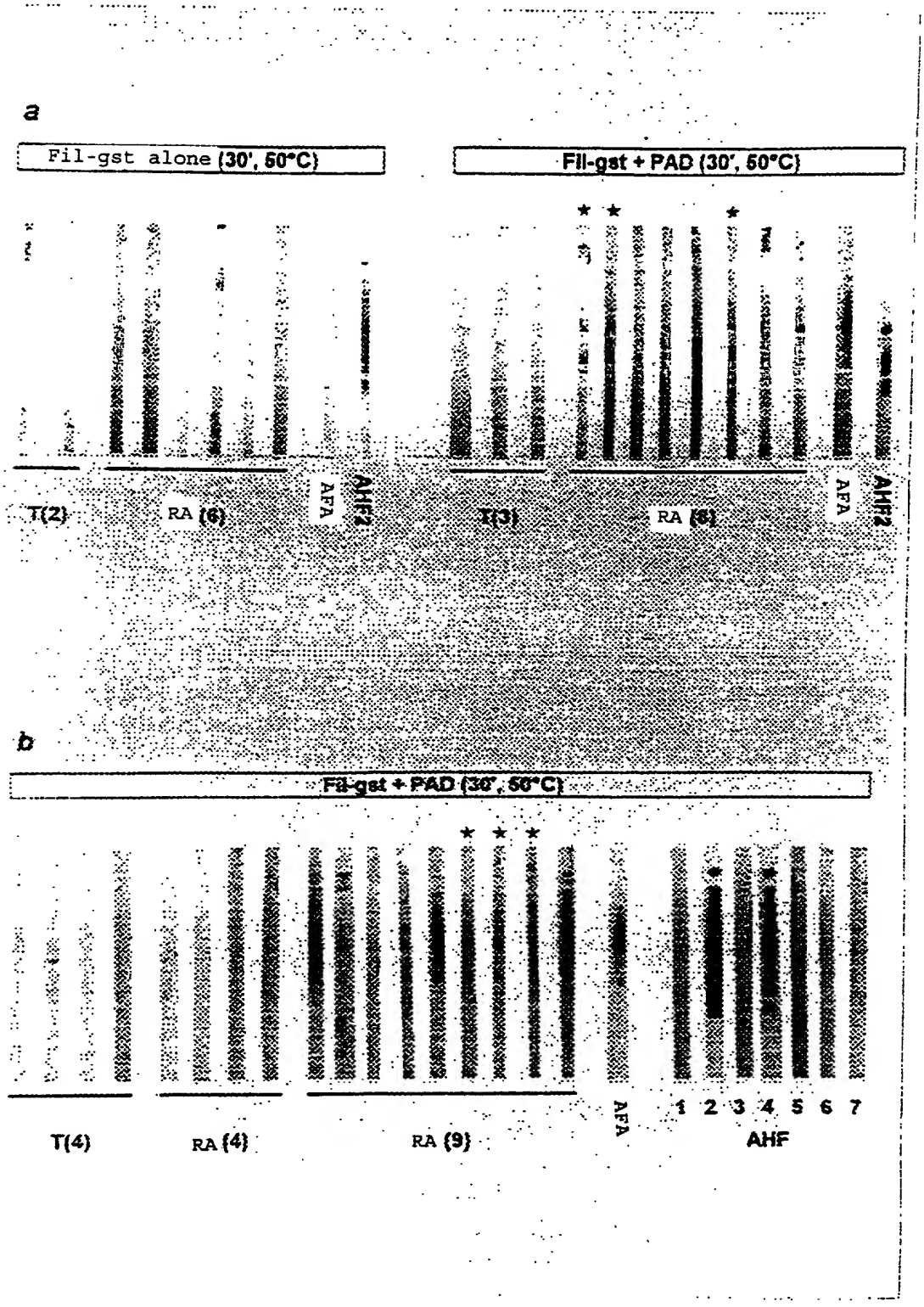
a
Fil-gst alone (30', 50°C)
Fil-gst + PAD (30', 50°C)
T(2)
RA (6)
AFA
AHF2
T(3)
RA (6)
AFA
AHF2
b
Fil-gst + PAD (30', 50°C)
T(4)
RA (4)
RA (9)
AFA
1 2 3 4 5 6 7
AHF

FIG.4

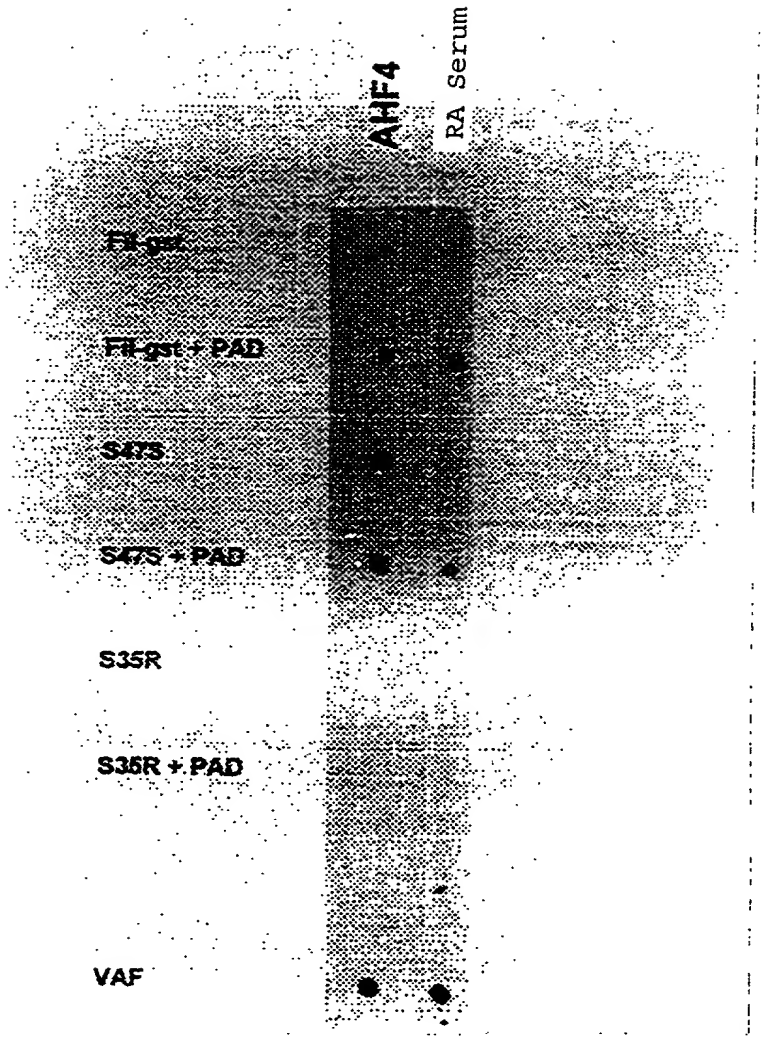
AHF4
RA Serum
Fil-gst
Fil-gst + PAD
S47S
S47S + PAD
S35R
S35R + PAD
VAF

FIG.5

Attorney's Docket No. 3339-392

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

(Foreign Agent Involved)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ANTIGENS DERIVED FROM FILAGGRINS AND THEIR USE FOR THE DIAGNOSIS OF RHEUMATOID ARTHRITIS,

the specification of which is attached hereto unless the following box is checked:

[X] was filed on Feb. 26, 1999 as United States Application Number or PCT International Attorney's Docket No.
Application Number 3339-392 and was amended on _____ (if applicable). (PCT/FR97/01541)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			**Priority Claimed**	
96 10651	FRANCE	August 30, 1996	[x] Yes	[ ] No
(Number)	(Country)	(Day/Month/Year Filed)		
_____	_____	_____	[ ] Yes	[ ] No
(Number)	(Country)	(Day/Month/Year Filed)		

I hereby claim the benefits under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

_____ _____
(Number) (Filing Date)

_____ _____
(Number) (Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C.; § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Appln. Serial No.)	(Filing Date)	(Status --patented/pending/aban.)
(Appln. Serial No.)	(Filing Date)	(Status --patented/pending/aban.)

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from my French representatives, Cabinet Ores, as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

I hereby appoint the practitioners associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

Customer Number 826

ADDRESS CORRESPONDENCE TO THE ATTENTION OF:	DIRECT ALL TELEPHONE CALLS TO:
Raymond O. Linker, Jr. Registration No. 26,419	Raymond O. Linker, Jr. Registration No. 26,419 Tel (704) 331-6000 Fax (704) 334-2014

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Residence:

Citizenship:

Post Office Address:

159780